



# Isolation methods of large and small extracellular vesicles derived from cardiovascular progenitors: A comparative study

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## ABSTRACT

Since the discovery of the beneficial therapeutical effects of extracellular vesicles (EVs), these agents have been attracting great interest as next-generation therapies. EVs are nanosized membrane bodies secreted by all types of cells that mediate cell–cell communication. Although the classification of different subpopulations of EVs can be complex, they are broadly divided into microvesicles and exosomes based on their biogenesis and in large and small EVs based on their size. As this is an emerging field, current investigations are focused on basic aspects such as the more convenient method for EV isolation. In the present paper, we used cardiac progenitor cells (CPCs) to study and compare different cell culture conditions for EV isolation as well as two of the most commonly employed purification methods: ultracentrifugation (UC) and size-exclusion chromatography (SEC). Large and small EVs were separately analysed. We found that serum starvation of cells during the EV collecting period led to a dramatic decrease in EV secretion and major cell death. Regarding the isolation method, our findings suggest that UC and SEC gave similar EV recovery rates. Separation of large and small EV-enriched subpopulations was efficiently achieved with both purification protocols although certain difference in sample heterogeneity was observed. Noteworthy, while calnexin was abundant in large EVs, ALIX and CD63 were mainly found in small EVs. Finally, when the functionality of EVs was assessed on primary culture of adult murine cardiac fibroblasts, we found that EVs were taken up by these cells, which resulted in a pronounced reduction in the proliferative and migratory capacity of the cells. Specifically, a tendency towards a larger effect of SEC-related EVs was observed. No differences could be found between large and small EVs. Altogether, these results contribute to establish the basis for the use of EVs as therapeutic platforms, in particular in regenerative fields.

## 1. Introduction

Extracellular vesicles (EVs) are nanosized particles secreted by all cell types that mediate intercellular communication [1]. They are delimited by a lipid bilayer and they contain a great variety of active biological molecules that reflect the cytosolic content of the source cell, such as proteins, lipids and nucleic acids [2]. EVs can be broadly

classified into three subtypes depending on their cellular origin: exosomes, microvesicles and apoptotic bodies; and in large and small EVs based on their size. Efforts directed towards exploiting EVs as drug delivery systems and therapeutic agents have experienced a huge increase in the last few years as they are attracting the interest of many scientists [3]. The potential of EVs relies on their exceptional advantages associated mainly with their biological nature. Upon secretion, they can reach

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distal cells through body fluids or act on nearby cells. In addition, EVs possess the ability to cross membrane barriers and deliver their functional cargo on recipient cells modulating their behaviour [4,5]. As therapeutic agents, they are being investigated as potential cell therapy substitutes for many diseases, which may help to overcome serious cell-associated risks such as teratomas or immune rejection [6,7]. Additionally, they may be used as therapeutic RNA carriers due to their natural capability to deliver RNA [8,9].

Despite their recognised beneficial effects, our knowledge about EVs is still limited since this field is in its infancy. Current investigations are focused on elucidating critical aspects such as the optimal isolation method, storage conditions, characterization protocols, therapeutic doses and delivery strategies [10,11]. Profound and exhaustive research of these issues is mandatory, as it would accelerate a potential clinical translation. For example, controversy remains surrounding the presence of serum in culture media during the EV collecting period [12] as the absence of serum could severely affect EV production and cargo content because of cell stress [13]. On the other hand, co-precipitation of EVs and proteins from the serum together with the EVs of interest is a major concern that needs to be carefully addressed [14]. Until recently, differential ultracentrifugation (UC) was the preferred technique for EV isolation. This method relies on the sedimentation of EVs after centrifugation at high speeds. However, there is now some suggestions that UC originates biophysical damage to EVs due to shearing forces and vesicle aggregation [15]. In view of this, a novel method for EV isolation was proposed based on ultrafiltration followed by size-exclusion chromatography (SEC) [16–18]. Although less widely explored than UC, related studies indicate that SEC leads to more intact EVs, a higher yield and higher functionality [19,20]. While the potential and therapeutic avenues opened by EVs are undeniable, further investigations are required to reach a consensus on gold-standard protocols for the isolation and characterization of pure and functional EVs.

In this study, we provide insights into fundamental aspects of working with EVs, namely the optimal cell culture conditions and isolation method for their subsequent application as therapeutic agents. Considering the potential delivery of EVs for heart repair, we analysed EV derived from cardiac progenitor cells (CPCs). Remarkably, this study includes the isolation and separate analysis of different subpopulations of EVs: large and small EVs, an area that is still quite unexplored in most studies. We found that the use of culture media supplemented with EV-free serum increased cell-derived EV yield compared to cell starvation. Additionally, SEC represented an effective isolation method for obtaining EV populations with an acceptable yield and purity and a tendency towards a higher functionality than UC. Finally, we show that isolated EVs are internalized by primary cardiac fibroblasts and inhibit their migration and proliferation.

## 2. Material and methods

### 2.1. Materials

CPCs were obtained from Coretherapix SLU (Madrid, Spain). Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), neurobasal medium, fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine, B-27, N-2, insulin-transferrin-selenium (ITS), phosphate-buffered saline (PBS) and Hank's balanced salt solution (HBSS) were purchased from Gibco (Carlsbad, CA, USA). Insulin-like growth factor II (IGF-II), endothelial growth factor (EGF) and basic fibroblast growth factor (bFGF) were obtained from Peprotech (London, UK). Mouse leukemia inhibitory factor (LIF) and 100 kDa amicon® ultra-15 spin filters were provided by Merck Millipore (Darmstadt, Germany). DMEM,  $\beta$ -mercaptoethanol, ECL™ Prime Western blotting detection reagents, glycerol, triton X-100, goat serum and PKH26 Red Fluorescent Cell Linker Kit were provided by Sigma (Barcelona, Spain). The 0.22  $\mu$ m polyethersulfone (PES) filters and 40  $\mu$ m cell strainer were obtained from Corning (Corning, NY, USA). qEV10/70 nm size-exclusion

chromatographic column was purchased from Izon Science (Oxford, UK). The 10% sodium dodecyl sulfate (SDS), 4x Laemmli buffer and 0.45  $\mu$ m pore-size nitrocellulose membrane were provided by BioRad (Madrid, Spain). Pierce™ BCA Protein Assay kit was obtained from ThermoFisher Scientific (Waltham, MA, USA). Mouse anti-ALIX primary antibody was obtained from BD Bioscience (Madrid, Spain), goat anti-CD63 and goat anti-calnexin primary antibodies from SICGEN (Cantanhede, Portugal), and rabbit anti-collagen type I primary antibody from Rockland Immunochemicals (Limerick, PA, USA). Sheep anti-mouse IgG HRP and mouse anti-goat IgG HRP were provided by GE Healthcare (Madrid, Spain) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Liberase TH was purchased from Roche (Barcelona, Spain). Donkey anti-rabbit Alexa Fluor 488, Novex™ Reversible Membrane Protein Stain Kit and RBC lysis buffer were provided by Invitrogen (Barcelona, Spain).

### 2.2. CPC culture

Isolation of CPCs from adult male Wistar rat hearts and their subsequent characterization was performed by Coretherapix SLU, as previously described [21]. Cells were seeded at a 3000–5000 cells/cm<sup>2</sup> density and cultured in low oxygen conditions (3% O<sub>2</sub>) using DMEM/F12 and neurobasal medium (1:1) supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 1% B-27, 0.5% N-2, 0.5% ITS, 50  $\mu$ M  $\beta$ -mercaptoethanol and the following growth factors: IGF-II (30 ng/ml), EGF (20 ng/ml), bFGF (10 ng/ml) and LIF (10 ng/ml). For the collection of EVs, cells were cultured in three different conditions: i) basal medium (only DMEM/F12 and neurobasal medium), ii) serum-free medium (DMEM/F12 plus all cytokines, no B-27, N-2 nor serum) or iii) 5% EV-depleted complete medium [DMEM/F12 plus B-27 and N-2 as complements and FBS that was previously ultracentrifuged 16 h at 100,000g to remove EVs (EVs-depleted FBS)]. For comparison of UC and SEC, CPCs were only cultured in the third condition described above. After 24 h, EV conditioned medium was collected and refreshed for another 24 h.

### 2.3. EV isolation

At 24 and 48 h, conditioned medium was harvested and EVs were isolated by UC or SEC. In both methods, a preliminary step was included to eliminate any cell or debris remaining in the conditioned medium that could result in misleading results or collapse the SEC column. To that end, the medium was centrifuged at 2,000g for 20 mins at 4 °C, the supernatant was collected and centrifuged again in the same conditions.

#### 2.3.1. Ultracentrifugation

For EV isolation by UC, a Beckman Optima™ LE-80 K ultracentrifuge with an SW 32 Ti rotor was used. First, the conditioned medium was centrifuged at 10,000g for 50 mins at 4 °C to obtain a large EV-enriched pellet. This pellet was further purified by an additional washing step by resuspension in filtered PBS and a second centrifugation at 10,000g for 70 mins at 4 °C. Pellet was resuspended in 50  $\mu$ l of PBS and stored at –80 °C. To isolate small EVs, large EV-free supernatant was filtered using 0.22  $\mu$ m PES syringe filters and ultracentrifuged at 100,000g for 3 h at 4 °C. Then, the pellet was washed with filtered PBS and ultracentrifuged again in the same conditions. Finally, the resulting small EV-enriched pellet was resuspended in 50  $\mu$ l of PBS and stored at –80 °C until further characterization.

#### 2.3.2. Size-exclusion chromatography

For EV isolation by SEC, harvested conditioned medium was concentrated to 10 ml by ultrafiltration using 100 kDa amicon® ultra-15 spin filters, before loading the sample into the pre-washed qEV10/70 nm chromatographic column. The eluent was collected in 5 ml fractions until all the samples had gone through the column. Finally, the EV elution profile was obtained by monitoring absorbance at 280 nm. EV-

enriched fractions were stored at  $-80^{\circ}\text{C}$  until further characterization.

## 2.4. EV characterization

### 2.4.1. Protein content

To estimate total protein content on EV samples obtained by UC and SEC, EVs were first lysed by incubation with lysis buffer (1% Triton X-100 + 0.1% SDS in  $\text{H}_2\text{O}$ ) for 30 mins on ice. Protein quantification was performed using the Pierce™ BCA Protein Assay kit following the manufacturer's indications. Samples were measured in triplicate and values were averaged for total protein estimation. For comparison of UC and SEC isolation methods, results were normalized by the number of cells.

### 2.4.2. Nanoparticle tracking analysis (NTA)

Particle number and size distribution in the different EV subpopulations were measured by NTA using a Nanosight NS300 (Malvern Panalytical, Almelo, The Netherlands) with a camera level set at 13 and a detection threshold at 5. Samples were measured in triplicate.

### 2.4.3. Transmission electron microscopy (TEM)

The size and morphology of large and small EVs were analysed by TEM with an FEI Tecnai™ transmission electron microscope. Previously, samples were adsorbed to a carbon-coated grid, fixed in 2% glutaraldehyde followed by negative staining using uranyl acetate. Finally, samples were embedded in uranyl-methylcellulose to increase the contrast of the membrane structure and allowed to dry.

### 2.4.4. Expression of specific markers and presence of serum proteins

For identification and characterization of the different EV subpopulations, western blot for ALIX, CD63 and calnexin was performed. For that, 3  $\mu\text{g}$  of EVs were lysed and protein content denatured using Laemmli buffer before loading in an SDS-PAGE gel for protein separation. Next, proteins were transferred to a nitrocellulose membrane, which was then blocked with 5% milk in TBS-Tween for 1 h and incubated overnight with corresponding primary antibody (anti-ALIX: 1:1000, anti-CD63: 1:5000 or anti-calnexin: 1:2500) at  $4^{\circ}\text{C}$  under agitation. On the next day, the membrane was washed three times with TBS-T and incubated with the corresponding IgG HRP secondary antibody (sheep anti-mouse or mouse anti-goat) (1:5000 and 1:2000, respectively). Finally, the membrane was incubated with ECL™ Prime Western blotting detection reagents for 5 mins and exposed using the ChemiDoc system (Bio-Rad, Madrid, Spain). The presence of proteins in the preparations was assessed using Novex™ Reversible Membrane Protein Stain Kit following the manufacturer's instructions.

## 2.5. Assessment of EV functionality

### 2.5.1. Isolation and culture of mouse cardiac fibroblasts

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Navarra and performed according to the requirements of the EU legislation.

Mouse cardiac interstitial cells were obtained from 8 to 10 week old mice, as previously described [22]. Briefly, after sacrifice, the thorax was opened and the heart was perfused with ice-cold PBS pH 7.6, atria were excised and tissues were placed in DMEM supplemented with 10% FBS on ice. Next, ventricles were minced using a sterile scalpel. Pieces of tissue were incubated in liberase TH (125  $\mu\text{g}/\text{mL}$ ) diluted in HBSS++ solution for 10 min at  $37^{\circ}\text{C}$  in an orbital shaker. After the enzymatic incubation, partially digested tissue was mechanically dissociated by slowly pipetting to reach a single cell suspension. The supernatant was filtered through a 40  $\mu\text{m}$  nylon cell strainer to discard cardiomyocytes. The digestion was repeated with the sedimented pieces and the supernatants were pooled together. The supernatant was centrifuged at 1500 rpm for 5 min and erythrocytes were removed using RBC lysis buffer. The total time for enzymatic digestion was 30 min. The final pellet was

resuspended in complete medium (DMEM + 10% FBS + 1% penicillin/streptomycin + 1% L-Glutamine) containing 10 ng/mL of bFGF and plated in a 0.1% (w/v) gelatin-coated 6-well-plate well (one heart per well). Cardiac fibroblasts were selected by attachment through washing the wells twice with PBS after overnight incubation and followed by a replacement of the complete medium plus 10 ng/mL of bFGF, lowering the concentration to 5 ng/mL (passage 1) and 2 ng/mL (passage 2) over time.

### 2.5.2. Uptake by cardiac fibroblasts

For cell uptake evaluation, large EVs were fluorescently labelled. Briefly, they were mixed with PKH26 and diluent C following the manufacturer's indications before incubation for 5 mins at room temperature (RT). Then, EV-free DMEM was added to the solution (1:1), mixed and incubated for an additional 5 mins at RT. Finally, EVs were centrifuged at 10,000 g for 30 mins and resuspended in 50  $\mu\text{L}$  of DMEM.

Adult primary cardiac fibroblasts in passage 2 were seeded in 8-well slide chambers at a density of  $7.5 \times 10^3$  cells/well. After 24 h, cells were subjected to starving conditions overnight. The next day, serum-free medium supplemented with 5  $\mu\text{g}/\text{mL}$  of stained large EVs obtained by UC and SEC or an equivalent amount of free PKH26 dye was added to the cells. At 3 h and 6 h, cells were fixed with paraformaldehyde 4% for 30 mins at  $4^{\circ}\text{C}$ , washed in PBS, permeabilized and blocked in SBT solution (16% goat serum, 1% bovine serum albumin and 0.1% Triton X-100) for 1 h at RT and incubated overnight with rabbit anti-collagen type I antibody (1:200) at  $4^{\circ}\text{C}$ . On the next day, slides were washed three times in PBS and incubated with donkey anti-rabbit Alexa Fluor 488 (1:200) for 1 h at RT. Finally, preparations were washed again, counterstained with DAPI, mounted using a mixture of PBS:glycerol (1:1) and visualized using an LSM 800 confocal microscope (Zeiss, Madrid, Spain). Two biological replicates were performed per condition per time point.

### 2.5.3. Evaluation of the anti-fibrotic effect

The functionality of EVs was investigated using a scratch assay. Adult cardiac fibroblasts in passage 2 were seeded in 24-well culture plates at a density of  $1.5 \times 10^4$  cells/well. After 48 h, cells were subjected to starving conditions overnight without bFGF. The next day, a straight line was generated on the monolayer with a pipette tip. Cells were washed twice with PBS to remove debris and serum-free medium supplemented with 100  $\mu\text{g}/\text{mL}$  of specific subpopulations of EVs was added to the cells. The control group was cultured in serum-free medium without EVs. The capacity of the cells to migrate and occupy the denuded area was tracked for 48 h using a time-lapse microscope Axio Observer Z1 (Zeiss, Madrid, Spain). The covered area was quantified using Fiji software (version 1.46). Between 3 and 4 biological replicates were performed per condition.

## 2.6. Statistical analysis

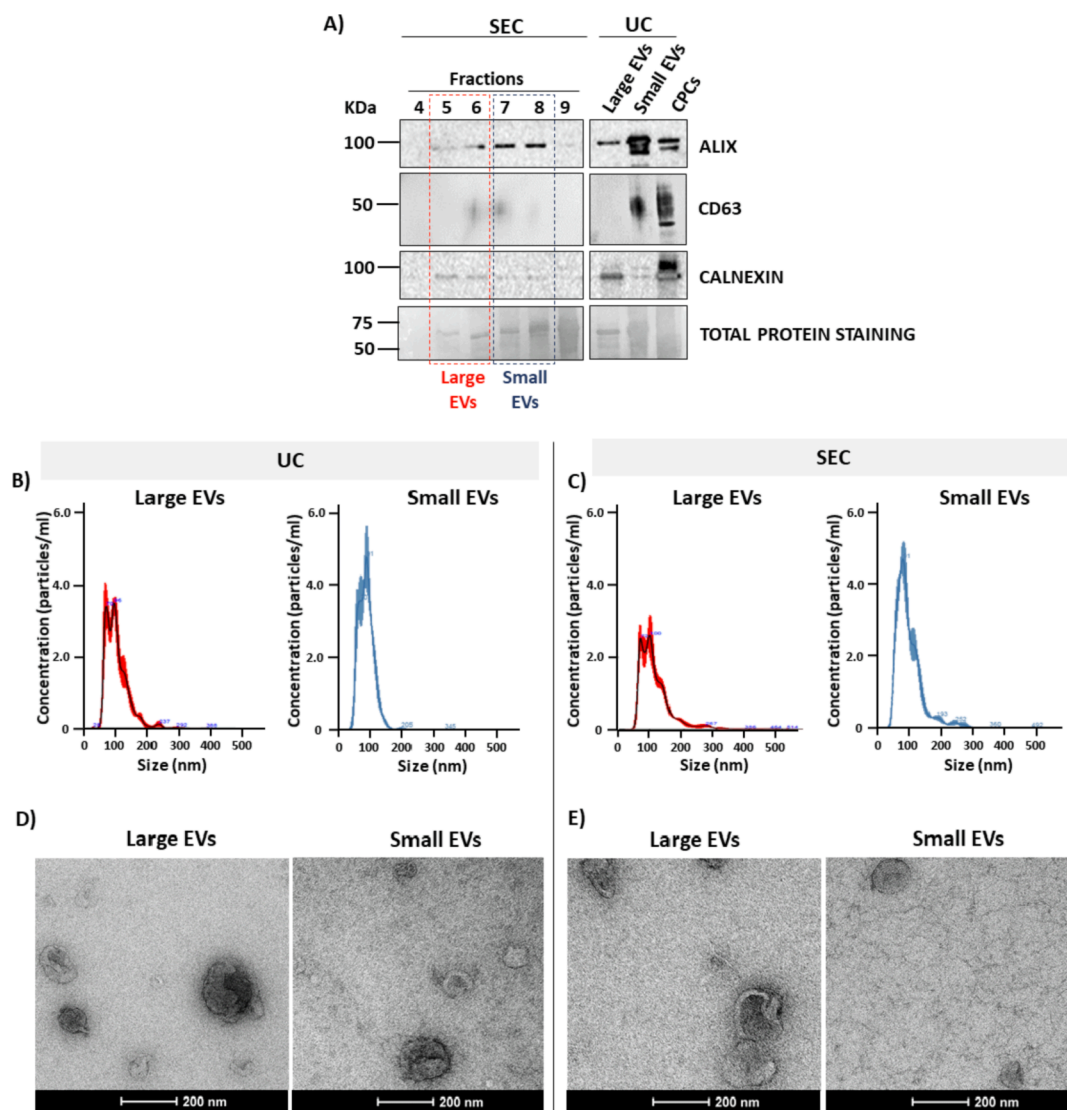
Statistical analysis was performed using GraphPad 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Differences between two groups were tested with student's *t*-test while differences among three or more groups were analysed using one-way ANOVA with Tukey post-hoc correction. Statistical significance was determined by *p* values  $< 0.05$ .

## 3. Results

### 3.1. EV characterization

#### 3.1.1. Large and small EVs differ in protein markers

First, the presence of specific markers of large and small EVs was analysed in EV preparations obtained by UC and SEC to characterize the different subpopulations. ALIX, a specific exosome marker, was present in the small EV sample obtained by UC and in fractions 7 and 8 of SEC (Fig. 1A). In this sense, ALIX was expected to appear at  $\approx 96$  kDa. However, an additional band corresponding to this protein ( $\approx 90$  kDa)



**Fig. 1. EV characterization.** A) Western blot analysis of the presence of ALIX, CD63 and calnexin in EVs obtained by UC and in the different SEC fractions, as well as total protein staining showing the co-purification of major serum proteins in the different EV-enriched preparations. B, C) Size distribution in large and small EV preparations isolated by UC (B) and SEC (C) measured by NTA. D, E) TEM images showing the morphology of large and small EVs obtained by UC (D) and SEC (E).

appeared in the UC sample but not in the SEC sample. Importantly, this marker could also be detected in the large EV preparation of UC and to a lesser extent in fractions 5 and 6 from SEC. Additionally, small EVs obtained with UC and EVs from fractions 6 and 7 of SEC were enriched in CD63. On the contrary, calnexin was present on large EVs both after isolation by UC and SEC (Fig. 1A), while it was almost undetectable in small vesicles. No large or small EV markers could be found in previous or subsequent SEC fractions, indicating that EVs were eluted in fractions 5 to 8. These results indicate that small EVs were characterized by the presence of ALIX and CD63, while large EVs were enriched in calnexin. Finally, qualitative assessment of the co-isolation of major serum proteins such as BSA was conducted by staining the nitrocellulose membrane with transferred proteins using Novex™ Reversible Membrane Protein Stain Kit. Equal protein amounts were loaded per lane. Staining showed different patterns of protein co-purification (Fig. 1A). As a control, a major presence of proteins was observed in SEC fraction 9 as serum proteins were expected to start eluting in this fraction. Remarkably, when both isolation methods were compared, UC led to a greater enrichment of a  $\approx 65$  KDa dense band, possibly corresponding to bovine serum albumin, especially in the small EV fraction compared to fractions 7 and 8 of SEC (Fig. 1A). Based on these results, we attributed fractions 5

and 6 from SEC to large EVs and fractions 7 and 8 to small EVs for further studies.

### 3.1.2. Large and small EV preparations show different vesicle enrichment

Size distribution was analysed by NTA. Large EV preparations showed a broad range of particles with sizes varying from 50 nm to 300 nm (Fig. 1B and C). By contrast, small EV samples presented a thin peak with smaller particles ranging from 30 nm to 150 nm, revealing a more homogeneous population. Small EVs obtained from SEC showed a minor population of larger particles in fraction 7 (Fig. 1C), a consequence of the gradual elution of small particles in this method. This population was absent in fraction 8 (data not shown). These results were confirmed by TEM. All samples showed EVs with spherical and cup-shaped morphology (Fig. 1D and E). While small EV preparations presented homogeneous particle sizes with very few vesicles larger than 150 nm, large EV preparations showed heterogeneity. These results reveal the homogeneity and reduced particle size in small EV samples in contrast to the more heterogeneous and larger vesicles found in the large EV-enriched preparations.



### 3.2. UC and SEC produce similar EV yield

Regarding the isolation method, EV recovery was similar ( $p > 0.05$ ) after isolation by UC or SEC (Fig. 2A). Interestingly, both isolation methods led to a greater recovery of small vesicles than large vesicles in terms of protein yield and number of particles, based on our definition of large and small vesicles. Specifically,  $5.7 \pm 0.9 \mu\text{g}$  of large EVs and  $20.9 \pm 4.0 \mu\text{g}$  of small EVs were obtained by UC compared to  $4.8 \pm 0.9 \mu\text{g}$  of large EVs (fractions 5 and 6) and  $16.7 \pm 3.4 \mu\text{g}$  of small EVs (fractions 7 and 8) that were obtained by SEC, for equal amounts of cells. When total EV particle number was quantified,  $1.5 \times 10^9 \pm 1.3 \times 10^8$  large EVs and  $4.8 \times 10^9 \pm 1.0 \times 10^9$  small EVs were obtained by UC. Similarly,  $1.7 \times 10^9 \pm 1.5 \times 10^8$  large EVs and  $2.1 \times 10^9 \pm 5.7 \times 10^8$  small EVs were found after SEC. In consonance, no significant differences were obtained in the ratio of particles per  $\mu\text{g}$  of protein (ratio range  $1.4 \times 10^8 - 2.9 \times 10^8$ ). These data may indicate that small vesicles are the predominant component of CPC-derived EVs and that UC and SEC allow for similar EV recovery.

In addition, protein yield and particle number were quantified in the different EV fractions for improved characterization of the method (Fig. 2B). Interestingly, EVs mostly eluted in fraction 6 ( $3.4 \pm 0.5 \mu\text{g}$  protein and  $1.2 \times 10^9 \pm 5.0 \times 10^7$  EVs) and in fraction 7 ( $5.7 \pm 0.6 \mu\text{g}$  protein and  $1.6 \times 10^9 \pm 1.9 \times 10^8$  particles). Few particles were found in fraction 5 ( $1.4 \pm 0.4 \mu\text{g}$  protein and  $3.6 \times 10^8 \pm 1.2 \times 10^8$  EVs) corresponding to the beginning of the large EVs elution. These fractions present similar purity with  $2.7\text{--}3.2 \times 10^8$  particles per  $\mu\text{g}$  of protein. Finally, although a large protein yield was obtained in fraction 8, particle number was low ( $9.4 \pm 1.2 \mu\text{g}$  protein and  $7.5 \times 10^8 \pm 2.1 \times 10^8$  EVs) indicating some contamination with serum proteins. In this fraction, the particle/protein ratio decreased to  $6.3 \times 10^7$ .

### 3.3. Starving culture conditions generates a dramatic decrease in EVs production

The production of EVs in different cell culture conditions was analysed as a determining factor compromising the final characteristics of the preparations and the efficiency of the process. Protein yield obtained by UC when CPCs were cultured in basal medium, serum-free medium or 5% EVs-free FBS complete medium were compared. At 24 h, a dramatic decrease in large and small EVs protein yield was observed when cells were cultured in the basal medium as well as in serum-free medium (Fig. 3A). The enhanced production of EVs by cells that received complete medium reached a 2.8- and 4.0-fold increase in the secretion of large EVs ( $p < 0.01$  and  $p < 0.001$ , respectively) and a 6.5- and 4.4-fold increase in the secretion of small EVs ( $p < 0.01$  for both) when compared with basal or serum-free conditions, respectively. These findings were critically exacerbated at 48 h with an insignificant amount of EVs isolated when medium lacked some or all components ( $p < 0.01$  and  $p < 0.05$  for large and small EVs, respectively, compared to complete medium) (Fig. 3B). By contrast, culturing in optimal conditions for an additional 24 h led to a 1.2- and 1.7-fold increase in large and small EVs production, respectively. In consonance, cell counting at 48 h revealed a decrease in the number of viable cells of 86% for basal medium and 68% for serum-free medium (Fig. 3C). These results suggest that medium composition severely influences EVs production. In this case, given the need to maximize the production of EVs, further studies were performed by culturing the cells in the presence of serum and all cytokines required.

### 3.4. CPC-derived EVs were taken up by primary cardiac fibroblasts

The functionality of specific EV subpopulations obtained by UC or SEC was analysed on primary cardiac fibroblasts. By confocal microscopy, we provided evidence that fluorescently labelled EVs were efficiently taken up by primary cardiac fibroblasts after 3 h of incubation (Fig. 4). In addition, vesicles were also detected embedded in cells

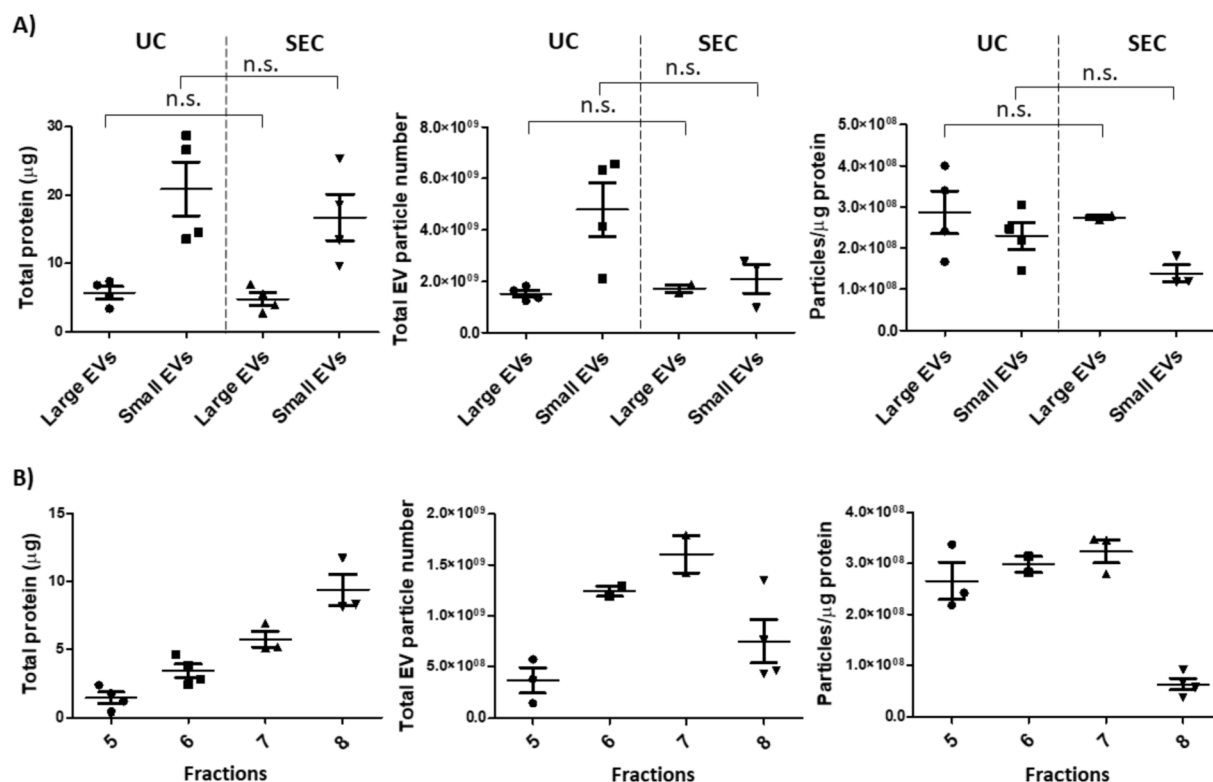
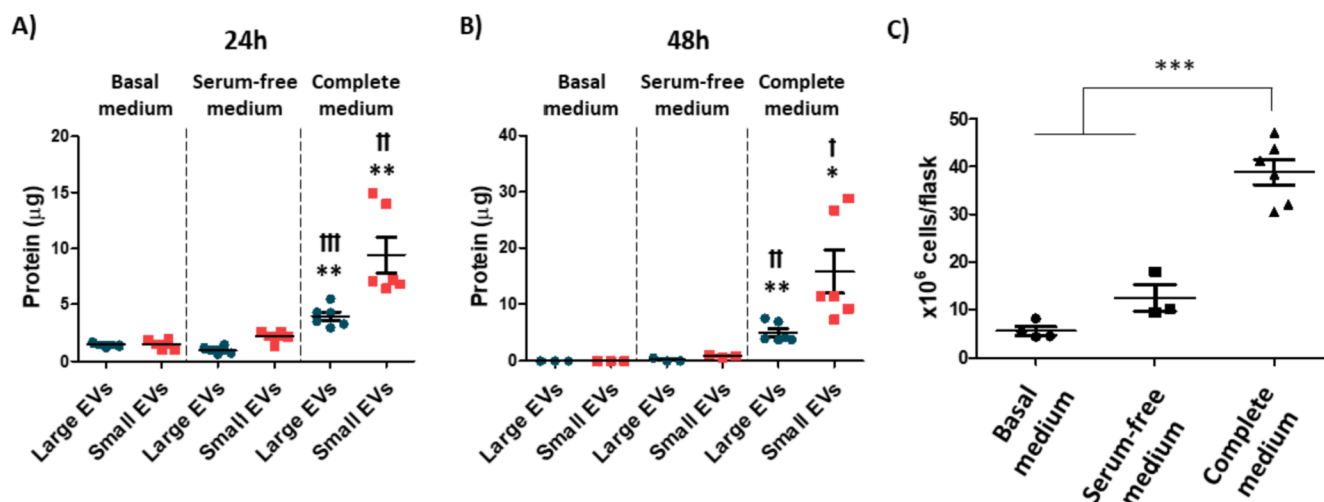


Fig. 2. Isolation efficiency with UC and SEC. Total protein, EV particle number and particles/ $\mu\text{g}$  protein were obtained for large and small EVs with UC and SEC (A) and with the different collected fractions in SEC (B). Data are mean  $\pm$  SEM. n.s.: non-significant.



**Fig. 3.** EV recovery yield using UC quantified as protein content in different media compositions. Protein yield obtained in the large EV- and small EV-enriched fractions after 24 h (A) and 48 h (B) of culturing CPCs in basal medium, serum-free medium or complete medium. C) Total cell count after 48 h of cell culture in basal medium, serum-free medium or complete medium. Data are mean ± SEM. \* p < 0.05 and \*\* p < 0.01 vs basal medium, † p < 0.05, †† p < 0.01 and ††† p < 0.001 vs serum-free medium, \*\*\* p < 0.001.

cytoplasm after 6 h, with no qualitative differences in vesicles internalization between both time points. On the other hand, no signal was found in cells alone or incubated with free PKH26 (Fig. 4). This observation reflects that EVs obtained from both methods are internalized by the cells, a finding that may indicate the vesicles preserved integrity.

### 3.5. CPC-derived EVs affect the proliferation and migration capacities of cardiac fibroblasts

After demonstrating that EVs secreted by CPCs are efficiently taken up by cardiac fibroblasts, the effect of specific subpopulations on the migration and proliferation of these cells was analysed by a scratch assay. Functional effects could be appreciated after 6 h of incubation. At this time, large EV-SEC ( $4.9 \pm 0.5\%$ ,  $p < 0.001$ ), small EV-UC ( $7.7 \pm 0.6\%$ ,  $p < 0.05$ ) and small EV-SEC ( $4.1 \pm 1.6\%$ ,  $p < 0.001$ ) samples showed a significant inhibition of wound closure compared to control ( $15.2 \pm 1.6\%$ ), while large EV-UC did not ( $9.2 \pm 1.9\%$ ) (Fig. 5A and B). This effect was maintained and further increased at 24 h, with more evident differences to control samples (control:  $46.6 \pm 7.3\%$ ; large EV-UC:  $30.4 \pm 3.8\%$ ; large EV-SEC:  $13.9 \pm 2.0\%$ ,  $p < 0.001$ ; small EV-UC:  $18.6 \pm 4.0\%$ ,  $p < 0.01$ ; small EV-SEC:  $12.8 \pm 3.1\%$ ,  $p < 0.001$ ). Finally, the largest differences were observed after 48 h with a dramatic prevention of fibroblast proliferation and migration of all tested EVs (large EVs-UC:  $41.6 \pm 0.5\%$ ,  $p < 0.05$ ; large EVs-SEC:  $20.5 \pm 4.9\%$ ,  $p < 0.001$ ; small EVs-UC:  $26.6 \pm 6.1\%$ ,  $p < 0.01$ ; small EVs-SEC:  $19.0 \pm 5.3\%$ ,  $p < 0.001$ ) compared to the control group, where the scratch was nearly closed ( $70.0 \pm 4.7\%$ ) (Fig. 5A and B, see online version for supplementary video 1). Although not significant, SEC samples showed a tendency towards a larger inhibition of fibroblasts biology compared to UC-derived EVs. No differences were found among large and small EVs. These results reveal that CPC-derived EVs possess anti-proliferative and migratory properties independently of their biogenesis or size and remain functional after isolation by UC and SEC.

## 4. Discussion

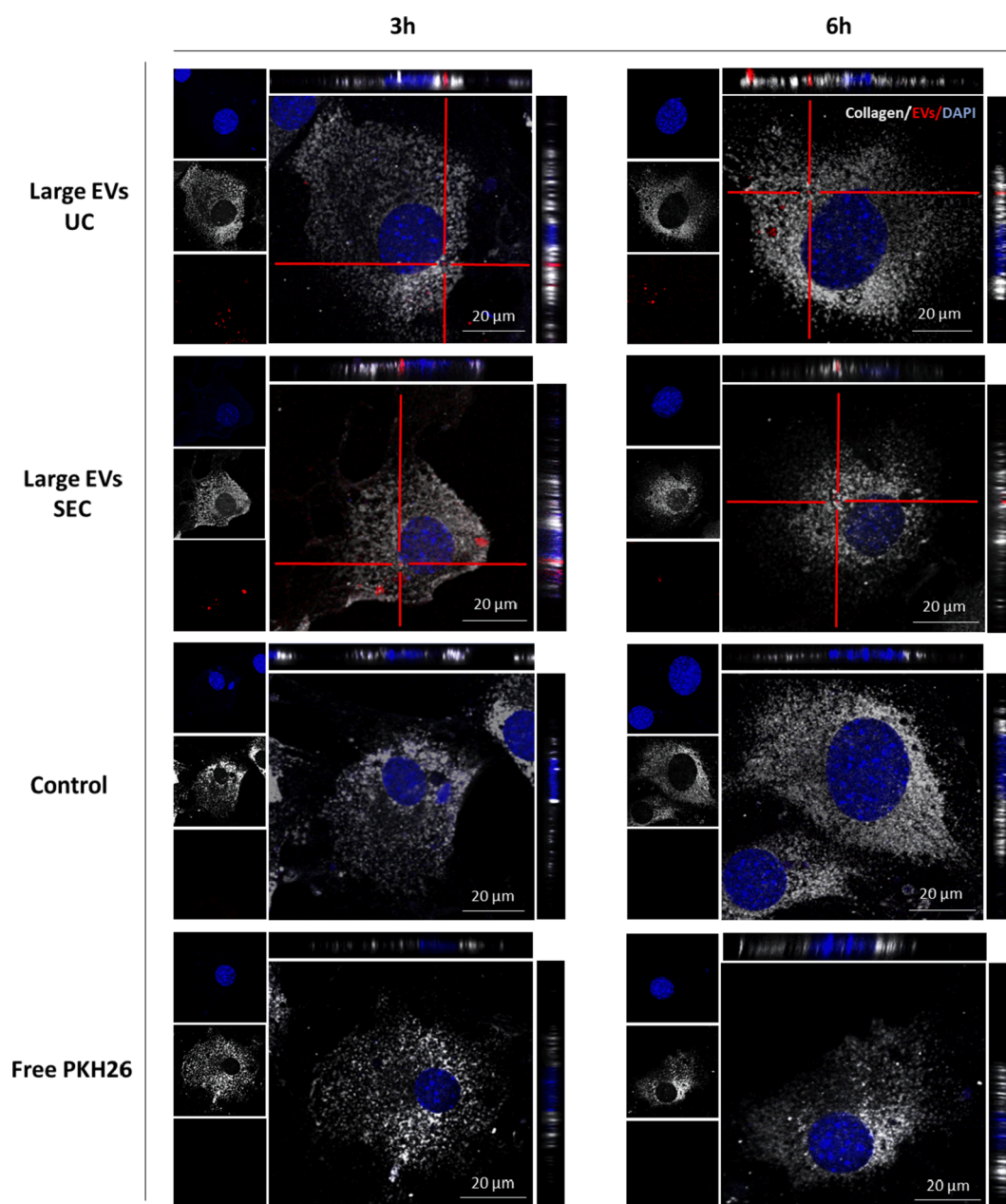
Encouraged by recent evidence of the potential of EV delivery for regenerative applications, many efforts are being directed towards the development of standardized protocols and techniques that facilitate EV translation. Within this framework, our research explores and provides insights into current uncertainties regarding EV purification, characterization and functionality, which could determine the efficacy and

success of the final strategy.

Although this paper constitutes a proof-of-concept for the isolation of EVs derived from one specific cell type, our results may be applicable to other cell sources. In addition, it is intended for a wide variety of medical applications, although the most immediate targeted application is cardiac repair. Therefore, CPCs were the cell source of choice for EV isolation. The selection of CPCs was founded on previous research articles from our group and others, supporting the multiple beneficial effects of these cells on cardiac repair, mainly by a paracrine effect, even 1-year post-administration [21,23,24]. Due to their cardiac origin, CPCs postulate as excellent candidates for heart repair purposes [25]. In addition, the use of EVs as therapeutic agents demands a high yield, a requirement that can only be achieved by relying on a cell source that allows easy expansion and culture conditions.

In this work, we sought to shed light on the isolation method that leads to optimal recovery, purity and functionality of EVs. We compared two of the most widely used protocols: UC and SEC. In the first approach, EVs were characterized by different techniques. Finding universal specific markers for different subclasses of EVs is challenging and should be carefully revised depending on the cell origin, culture conditions or cell state. Nowadays, there are no markers established to differentiate between large and small EVs [26]. Our analysis of EV markers revealed the presence of ALIX and CD63 in fractions enriched with small vesicles, as commonly found in other papers [27], and calnexin in those fractions enriched in larger vesicles. Interestingly, we found only one dense band corresponding to ALIX after SEC isolation while an additional band at a lower molecular weight was found after UC isolation. It is not the first time that this phenomenon has been described and this may be associated with different phosphorylation states [19]. However, it should be kept in mind that, in line with previous authors, the classification of EV subtypes based on the presence of certain proteins could be a mistake and other complementary techniques should be addressed [11]. Overall, we suggest that careful analysis of EV markers should be performed to allow/enable insights into the specific characteristics of EVs but without falling into the error of categorising vesicles based only on typically used markers.

One of the main purposes of the present study was the separation of large and small EVs for subsequent analysis. Despite the successful obtainment of large EV- and small EV-enriched preparations, size distribution analysis, TEM and western blot revealed a certain sample heterogeneity. Large EV samples were enriched in particles of a larger size than small EV samples, as had been initially expected, but they also



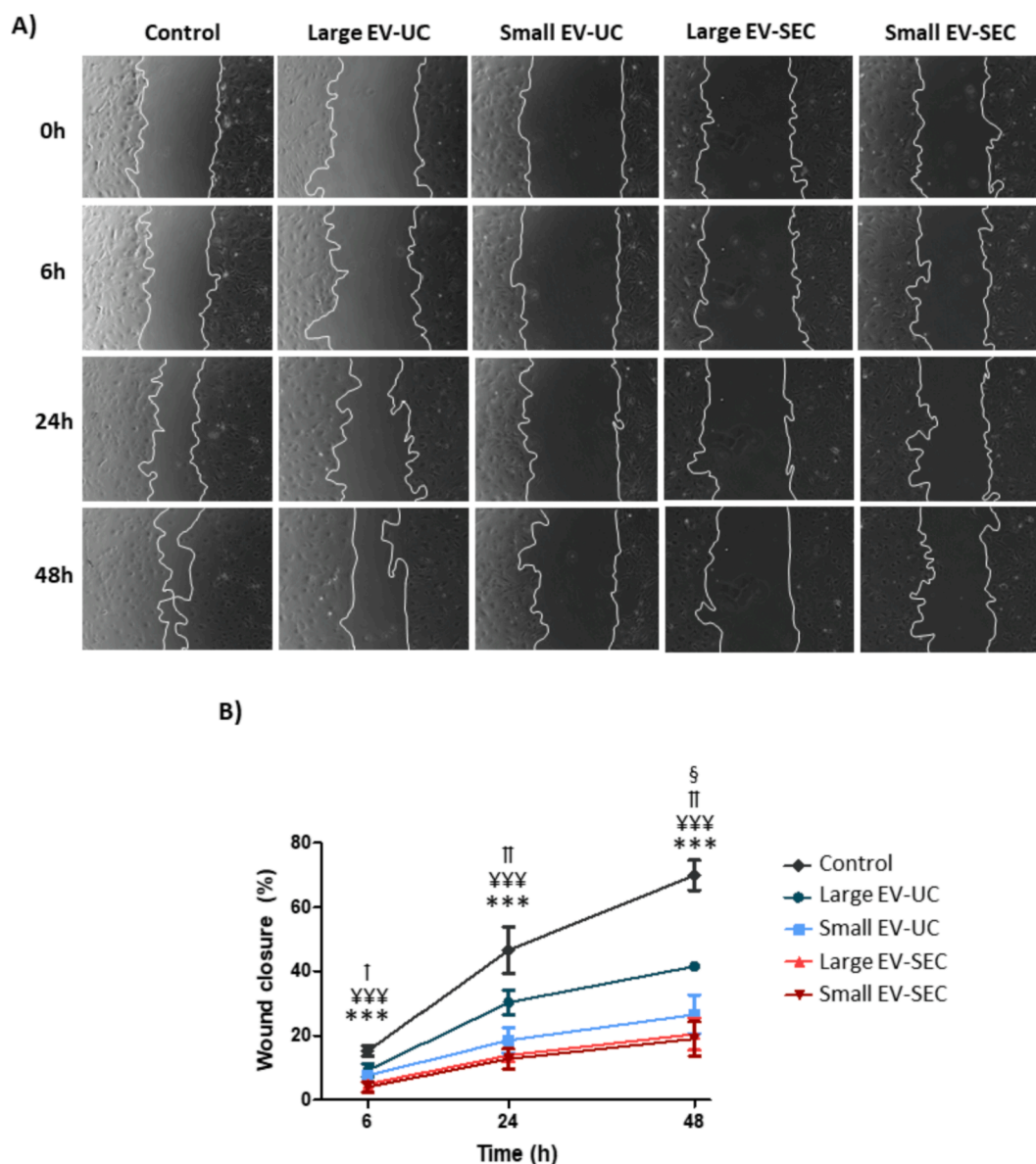
**Fig. 4.** EVs were taken up by cardiac fibroblasts. Individual channels for nuclei (DAPI, blue), cardiac fibroblasts (anti-collagen, white) and large EVs (PKH26, red), merge images and ortho view of Z-stack confocal images showing the internalization of EVs at 3 and 6 h. No signal was detected either in cells without EVs or cells incubated with free PKH26. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

presented small vesicles and expressed their characteristic markers. This is a common finding in other publications [28,29]. In addition, we should keep in mind that the general classification into large and small EVs could be too simple, given the diversity in EV subpopulations [28].

In a second approach, both isolation methods were compared. In good agreement with a study by Mol et al. [19] and in contrast to Nordin et al. [17], we found similar EV recovery with both methods, with small vesicles arising as the predominant subpopulation. Furthermore, the purity of EV samples has been described to vary between different isolation techniques. Co-isolation of free proteins and serum EVs remains a major concern linked to some protocols. The heavy presence of non-desired proteins gains special importance for downstream analysis, where non-EV molecules could lead to confusing results [30]. Besides, robust protocols for EV purification are needed in clinical applications to deliver precise doses, determine the most efficient EV source and provide strict control of product quality. Previously, Corso et al. verified

that SEC isolation enables the efficient separation of EVs from free proteins and RNAs [31]. In this sense, we found a similar particle/protein ratio with SEC and UC, which could be described as an indicator of sample purity. However, a great difference in sample purity was observed between fractions 7 and 8 from SEC. In this sense, SEC allows choosing between maximizing EV recovery or purity by collecting and pooling different fractions. As shown by the quantification of total particles and proteins in the different SEC fractions, the latest EV fraction co-eluted with a large number of proteins. If this fraction is discarded, it is possible to obtain small EVs with higher purity, as we observed after staining the membrane for total protein. Additionally, the use of longer SEC columns than the ones used in this work could provide a more precise separation of large and small EVs and decreased co-purification of other proteins. However, a deeper study, comparing the molecular composition of the different fractions, may clarify this issue.





**Fig. 5.** EVs showed anti-proliferative and anti-migration properties. A) Representative bright-field images showing the evolution of wound closure at 0, 6, 24 and 48 h in the control and EV groups. B) Quantification of wound closure percentage at 6, 24 and 48 h from the initial wound area in the different groups. Data are mean  $\pm$  SEM. Statistical differences from control group are: \*\*\*  $p < 0.001$  vs small EVs-SEC; ¥¥¥  $p < 0.001$  vs large EVs-SEC; †  $p < 0.05$  and ††  $p < 0.01$  vs small EVs-UC; and §  $p < 0.05$  vs large EVs-UC.

Next, the methodological inconsistency detected in the current literature led us to perform an evaluation of several crucial issues regarding the work with EVs [32]. To avoid co-isolation of EVs from serum or enrichment in contaminant proteins that generate misleading results, several previous papers describe serum deprivation of cells for EV collection [33,34]. This aspect takes on special relevance in EVs isolated by SEC, since many chromatographic columns are not compatible with serum samples. While the results may be highly dependent on cell type, we found that starvation of cells provoked a dramatic decrease in EVs production accordingly to previously reported data [35]. This issue could be attributed to cell death but also to changes in cell behaviour. Although this is beyond the scope of this paper, it appears that an abrupt change in culture conditions will probably be reflected in alterations in cell metabolism and EV cargo due to major cell stress [11]. Alternatively, as the International Society for Extracellular Vesicles (ISEV) guidelines recommend, we decided to implement a protocol for the removal of bovine serum EVs and proteins based on overnight UC followed by filtration [36].

Finally, we assessed and compared the functionality of EVs on primary cardiac fibroblasts based on previous literature demonstrating the anti-fibrotic properties of CPCs [37]. Cardiac fibrosis is associated with several biological processes, such as the migration and proliferation of fibroblasts, which ultimately results in the formation of a non-contractile scar. In this paper, the effect of EVs in both processes has been evaluated [38]. The functional properties of EVs observed in this research, as well as in other studies [39,40], confirms that CPCs exert this anti-fibrotic effect through paracrine secretion. Although no significant differences were observed between UC and SEC, the latter tend to originate a larger inhibition of fibroblasts migration and proliferation. In consonance, EVs were taken up by cardiac fibroblasts in as little as 3 h, confirming that EVs are responsible for the effect observed in fibroblasts. Based on this tendency, further experiments in other cell types or with different endpoints could reveal functional differences between the two methods. Regarding EV subpopulations, as seen in this study, both subpopulations could be useful for future cardiac repair studies.



## 5. Conclusions

This study explores how different cell culture conditions and isolation methods affect CPC-derived EV yield, characteristics and functionality. First, the culture of CPCs in different media revealed that the presence of serum in conditioned media is unavoidable for obtaining EVs with an acceptable yield. Furthermore, we showed that SEC is as reliable as UC, the gold standard method, for isolating EVs. Both methods rendered a similar separation of large and small EVs, showing that the latter is the predominant component of secreted EVs. Specific characterization of EV subpopulations showed similar protein markers after both isolation methods. While calnexin was abundant in the large EV-enriched fraction, ALIX expression was enhanced in the small EV-enriched fraction. The integrity of EVs was assured after isolation by TEM as well as by their internalization by cardiac fibroblasts. As a result, EVs inhibited the migration and proliferation of these cells, suggesting a potential anti-fibrotic capacity. A slightly superior functionality was observed after SEC isolation but no differences could be appreciated among subpopulations. Further studies will focus on evaluating EV efficacy in a preclinical myocardial infarction model.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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